Identification of Grass-specific Enzyme That Acylates Monolignols with p-Coumarate[*][S]

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Background: In grasses, monolignols can be enzymatically preacylated and incorporated into lignin with unknown effects on lignin function.

Results: The grass-specific acyltransferase enzyme OsPMT, expressed in *Escherichia coli*, acylated monolignols with *p*-coumarate.

Conclusion: OsPMT likely encodes an enzyme responsible for acylation of monolignols in grasses.

Significance: The identification of enzymes responsible for monolignol acylation allows for the assessment of lignin acylation.

Lignin is a major component of plant cell walls that is essential to their function. However, the strong bonds that bind the various subunits of lignin, and its cross-linking with other plant cell wall polymers, make it one of the most important factors in the recalcitrance of plant cell walls against polysaccharide utilization. Plants make lignin from a variety of monolignols including *p*-coumaryl, coniferyl, and sinapyl alcohols to produce the three primary lignin units: *p*-hydroxyphenyl, guaiacyl, and syringyl, respectively, when incorporated into the lignin polymer. In grasses, these monolignols can be enzymatically preacylated by *p*-coumarates prior to their incorporation into lignin, and these monolignol conjugates can also be "monomer" precursors of lignin. Although monolignol *p*-coumarate-derived units may comprise up to 40% of the lignin in some grass tissues, the *p*-coumarate moiety from such conjugates does not enter into the radical coupling (polymerization) reactions of lignification. With a greater understanding of monolignol *p*-coumarate conjugates, grass lignins could be engineered to contain fewer pendent *p*-coumarate groups and more monolignol conjugates that improve lignin cleavage. We have cloned and expressed an enzyme from rice that has *p*-coumarate monolignol transferase activity and determined its kinetic parameters.

Lignin is an important polymer in plant cell walls, providing structural support to cells, strength to stems, a waterproof lining to the vascular system, and many other functions. Plants make lignin from a variety of monomers, but lignin is generally considered to arise primarily from the three monolignols, *p*-coumaryl alcohol **1H**, coniferyl alcohol **1G**, and sinapyl alcohol **1S** (Fig. 1), which produce *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively, when incorporated into

the lignin polymer. These monolignols are synthesized from a branch of the phenylpropanoid pathway, where *p*-coumaric acid is activated by 4-coumarate: CoA ligase (4CL) to form *p*-coumaroyl-CoA **2a** (Fig. 2) (1). The CoA esters caffeoyl-CoA **2b** and feruloyl-CoA **2c** are derived from *p*-coumaroyl-CoA **2a**. Each of these CoA esters can then be reduced, first by cinnamoyl-CoA reductase and then by cinnamyl alcohol dehydrogenase, to the monolignols **1**. Additional pathways are possible; for example, the 4CL enzyme can activate caffeic and ferulic acids (data not shown) to form their CoA esters (2, 3). Once the monolignols are synthesized, they are transported to the cell wall, oxidized, and polymerized into lignin via radical coupling reactions (4, 5).

Lignins in nature are often found to be naturally partially acylated. It is now reasonably well established that acylated lignins arise from preacylated monomers rather than from acylation of the polymer (6–8). Studies indicate that monolignols can be enzymatically preacylated by various acids including: acetates, which occur at low levels in hardwoods but at high levels in palms, kenaf, abaca, and sisal (6, 9–11); *p*-hydroxybenzoates in palms and *Populus* species (willow, aspen, poplar) (12–18); and *p*-coumarates in both C3 and C4 grasses (19–22), prior to their incorporation into lignin. The function of such acylation is essentially unknown, but these monolignol conjugates can also be "monomer" precursors of lignin (23). These acylated monolignols, or monolignol conjugates, are then incorporated into the lignin polymer by polymerization and co-polymerization with the traditional monolignols, resulting in lignins that are (partially) acylated at their γ-positions (24). With *p*-coumarate levels being as high as 18% of corn lignin (20), acetylation of kenaf lignins being as high as 60% in kenaf (9), and apparently as high as 80% in agave (25, 26), total acylation (by *p*-coumarate and acetate) as high as 85% in abaca (25), and as monolignol *p*-coumarate-derived units may comprise up to 40% of the lignin in some grass tissues, it is evident that these monolignol conjugates can comprise a very high fraction of the monomer pool for those lignins. This acylation is found predominantly on syringyl units, suggesting that sinapyl alcohol rather than coniferyl alcohol is the primary monomer used for
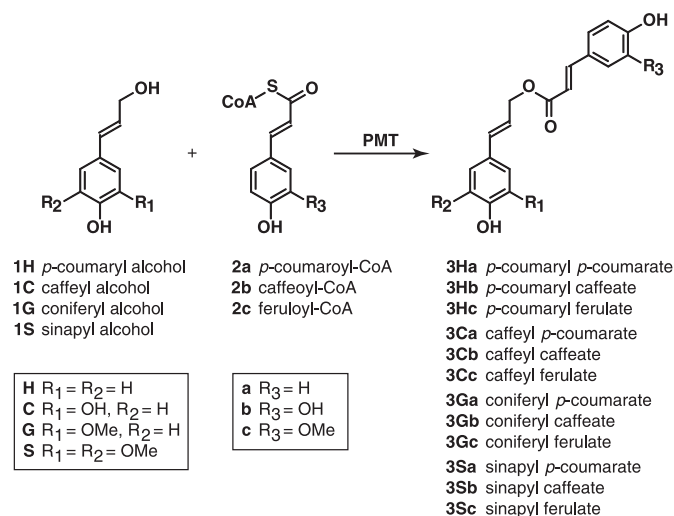
FIGURE 1. **Conjugates 3** from monolignols **1** and hydroxycinnamoyl-CoA thioesters **2.**

acylation and then lignification. Approximately 10% of *p*-coumarate can be found on guaiacyl units in maize (27, 28). *p*-Hydroxybenzoates and acetates have been found almost exclusively on syringyl units (6, 7, 11, 24).

p-Coumaroylation in grasses has been hypothesized as a way to produce sinapyl alcohol and lignin syringyl unit radicals via peroxidases that otherwise oxidize such substrates slowly (24). *p*-Coumarates are oxidized to the phenolic *p*-coumarate radical rapidly by most peroxidases; however, the *p*-coumarate moiety does not typically undergo radical coupling reactions *in planta* and remains almost entirely as terminal units with an unsaturated side chain and a free phenolic group (20, 29). We now know that when other phenolics are present, radical transfer (or radical exchange) from the *p*-coumarate radical to sinapyl alcohol or syringyl units (producing more stable radicals) is favored (30, 31). Lignification with sinapyl *p*-coumarate ester conjugates produces an essentially normal lignin "core" simply with pendant *p*-coumarate groups; no new bonds in the backbone of the lignin polymer arise from the radical coupling reactions (although postcoupling rearomatization reactions of β-β– coupled units must proceed via a different pathway than internal trapping by the γ-OH when the γ-OH is acylated) (6–8), and the resulting lignin backbone is not significantly more easily cleaved.

The pathways, and the required enzymes and genes by which monolignol conjugates are synthesized, are becoming important to delineate. First, as noted above, we have little knowledge of the function of lignin acylation. The ability to down-regulate acylation in plants that have acylated lignins, and to possibly introduce acylated lignins into plants that do not, should help shed some light on the roles and consequences of acylation. For example, with the gene responsible for the enzyme involved in biosynthesizing monolignol *p*-coumarate conjugates, grass lignins could be engineered to contain fewer pendent *p*-coumarate groups. Also, in a related project, our group has been interested in introducing monolignol ferulate conjugates, analogs of monolignol *p*-coumarates, into lignins in an attempt to introduce readily cleavable linkages into the backbone of the poly-

mer, facilitating lignin depolymerization in pulping or biomass pretreatments to reduce energy requirements (24, 32).

Currently, we do not know which enzymes are responsible for the biosynthesis of the monolignol conjugates. We expect candidates for this type of activity to be part of the BAHD acyltransferase family, which have been shown to acylate diverse substrates (33), including the acetylation of monolignols for use in isoeugenol synthesis (34). We have also established that *p*-coumaroylation in maize utilizes transferases that act on monolignols and *p*-coumaroyl-CoA (35). Here, we report on a gene from *Oryza sativa* that expresses a BAHD acyltransferase that catalyzes the acylation of monolignols **1** with *p*-coumarate, via *p*-coumaroyl-CoA **2a** (Fig. 1). This gene is found only in grasses and is co-expressed with genes involved in monolignol biosynthesis in rice (supplemental Table I) (36). We therefore suggest that this gene is implicated in the acylation of monolignols by *p*-coumarate, producing the monolignol conjugates **3** (*p*-coumaryl *p*-coumarate **3Ha**, coniferyl *p*-coumarate **3Ga**, and sinapyl *p*-coumarate **3Sa**) that are involved in lignification in grasses. Identifying the enzyme responsible for acylating grass lignins will help us to determine the function of this abundant modification.

EXPERIMENTAL PROCEDURES

Gene Synthesis—OsPMT[2] from *O. sativa* was submitted to Blue Heron Bio (State) for *Escherichia coli* codon optimization, synthesis, and cloning into the entry vector pENTR221 (Invitrogen). An expression clone containing an N-terminal His$_6$ tag was made by incorporating OsPMT into pDEST17 (Invitrogen) using Invitrogen Gateway cloning technology according to the manufacturer's guidelines.

Expression of OsPMT in E. coli and Purification—Cultures of BL21 cells (Invitrogen) containing the OsPMT expression clone were grown to an A_{600} between 0.4 and 0.5, cooled to 18 °C, and expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (Roche Applied Science). After 18-h (overnight) incubation at 18 °C, cells were harvested by centrifugation and frozen at −80 °C. The pellets from a 1-liter culture were suspended in 20 ml of binding buffer (20 mM Tris-HCl, pH 8, 0.5 M sodium chloride, 1 mM 2-mercaptoethanol), and cells were lysed using a French pressure cell press. The extract was then centrifuged at 50,000 × g for 30 min at 4 °C to separate soluble and insoluble protein fractions. Soluble protein was collected, and the pellet was suspended in 10 ml of 20 mM Tris-HCl, pH 8. Both fractions were analyzed for expression on an SDS-polyacrylamide gel by comparing bands of the expected molecular mass from an uninduced culture to the induced culture.

His-tagged OsPMT was purified by immobilized metal affinity chromatography (IMAC) using an AKTA purifier (GE Healthcare) operated with UNICORN 5.11 work station (GE Healthcare) and a protocol modified from the manufacturer's guidelines. Four stacked 5-ml HiTrap desalting columns (GE Healthcare) were equilibrated with binding buffer. A 5-ml ali-

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[2] The abbreviations used are: OsPMT, *Oryza sativa p*-coumarate monolignol transferase; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); IMAC, immobilized metal affinity chromatography; MS/MS, tandem MS; UPLC, ultra-HPLC.
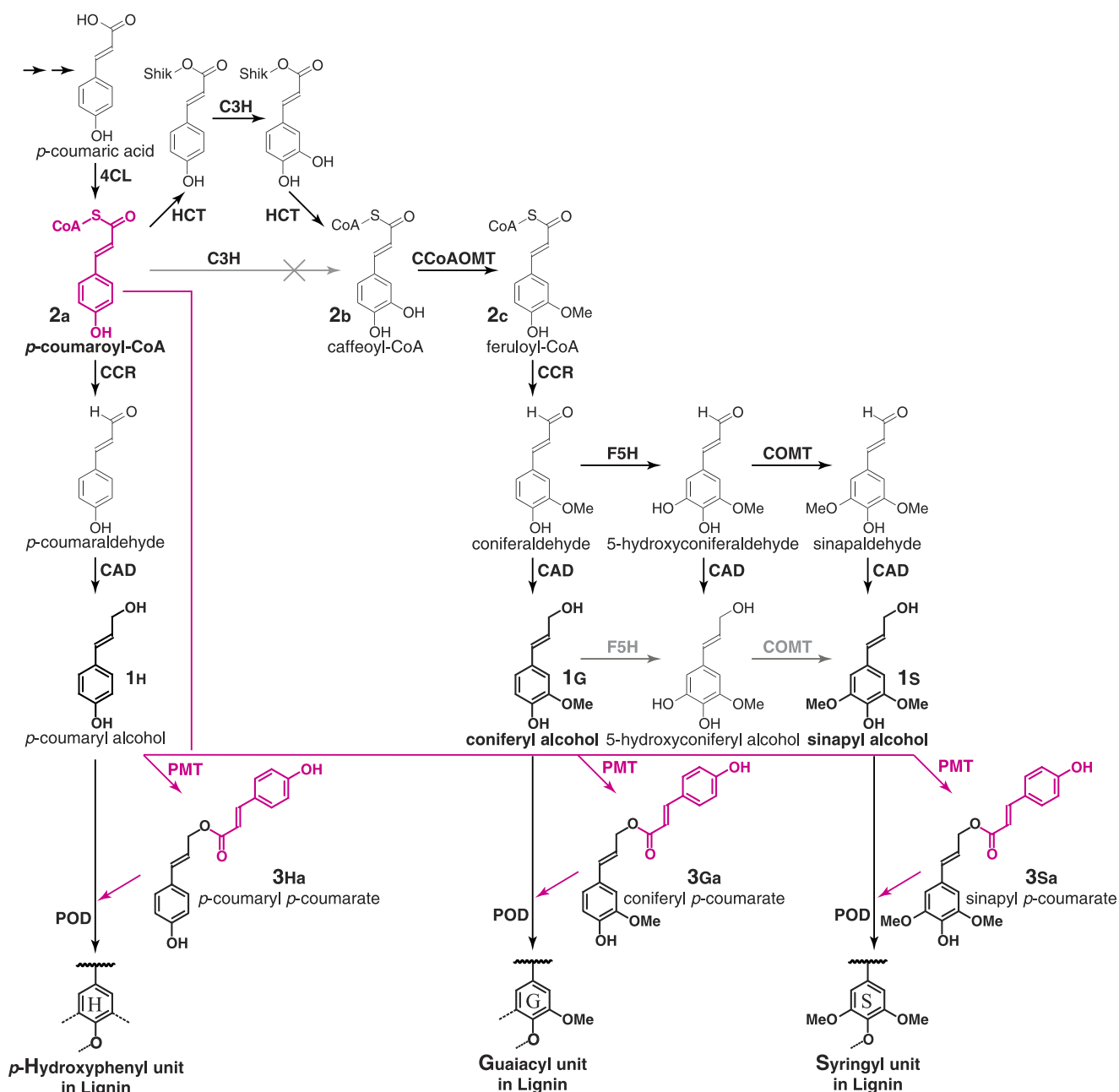
FIGURE 2. **Standard lignin biosynthetic pathway in angiosperms, adapted from Vanholme *et al.* (1), also showing putative pathways for synthesis of monolignol *p*-coumarate conjugates 3.** The predominant route toward the three main monolignols **1** is shown, with some of the more minor pathways in *gray*. The various routes through the pathway have been reviewed (2, 3). *4CL*, 4-coumarate:CoA ligase; *HCT*, *p*-hydroxycinnamoyl-CoA:quinate shikimate *p*-hydroxycinnamoyl transferase; *C3H*, *p*-coumarate 3-hydroxylase; *CCoAOMT*, caffeoyl-CoA *O*-methyltransferase; *CCR*, cinnamoyl-CoA reductase; *F5H*, feru-late/coniferaldehyde 5-hydroxylase; *COMT*, caffeic acid/5-hydroxyconiferaldehyde *O*-methyltransferase; *CAD*, cinnamyl alcohol dehydrogenase; *POD*, a generic peroxidase (generating the radicals required for monomer polymerization to lignin). Compound numbers are from Fig. 1.

quot of the soluble protein was injected onto the desalting column and eluted with binding buffer at a flow rate of 1 ml/min. Fractions with the highest protein concentrations, as indicated by UV absorbance, were collected in 1-ml fractions. These combined fractions were applied to a 1-ml HiTrap HP column (GE Healthcare), charged with Ni^{2+}, and conditioned with binding buffer, at a flow rate of 0.2 ml/min. The column was washed with 5 ml of buffer A (20 mM Tris-HCl, pH 8, 0.5 M sodium chloride, 1 mM 2-mercaptoethanol, and 20 mM imidazole), then bound protein was eluted at 1 ml/min over a 20-ml linear gradient from buffer A to buffer B (20 mM Tris-HCl, pH 8,

0.5 M sodium chloride, 1 mM 2-mercaptoethanol, and 500 mM imidazole). Fractions containing protein were collected and analyzed by SDS-PAGE; bands of the expected size were extracted from the SDS-polyacrylamide gel and sent to the MSU Proteomics Core for in-gel trypsin digestion followed by LC-MS/MS. Peptides were searched against the *O. sativa* genome data base (NCBI) and identified by Mascot. IMAC fractions with the highest concentration of OsPMT were combined and further purified by size exclusion chromatography using a Superdex 75 10/300 GL gel filtration column (GE Healthcare) and exchanged into a pH 6 buffer containing 100 mM sodium

phosphate. Protein samples were concentrated to 1 μg/μl in 100 mM sodium phosphate, pH 6, containing 100 ng/μl BSA (NEB) and a complete mini EDTA-free protease inhibitor tablet (Roche Applied Science) using an Amicon Ultracel 10K membrane filter (Millipore).

Enzyme Activity Assay—The CoA thioesters, *p*-coumaroyl-CoA **2a**, caffeoyl-CoA **2b**, and feruloyl-CoA **2c**, used as substrates in the OsPMT enzyme assay, were synthesized using the tobacco 4-coumarate CoA-ligase (4CL) with a C-terminal His tag in the vector pCRT7/CT TOPO (provided by Eran Pichersky, University of Michigan). These CoA thioesters were purified using Sep-Pak cartridges (Waters Corporation) following a method modified from Beuerle and Pichersky (37). The concentration for each CoA thioester was calculated based on its absorbance maximum and extinction coefficient (37, 38). Ferulic acid, caffeic acid, *p*-coumaric acid, and acetyl-CoA were purchased from Sigma-Aldrich. Purified CoA thioesters were analyzed for purity using an Acquity Ultra Performance LC with an Acquity UPLC BEH C18 1.7 μm 2.1 $\times$100-mm column and the Acquity Console and Empower 2 Software (Waters Corporation).

Authentic coniferyl *p*-coumarate **3Ga** and sinapyl *p*-coumarate **3Sa** were synthesized as described previously (39). *p*-Coumaryl *p*-coumarate **3Ha** and sinapyl acetate were made by an analogous route (39).

The OsPMT enzyme activity assay, in 50 mM sodium phosphate buffer, pH 6, containing 1 mM dithiothreitol (DTT), 1 mM CoA thioester, 1 mM monolignol, and deionized water to produce a final volume of 50 μl, was initiated by adding of 1 μg of purified PMT protein in 1$\times$ BSA (NEB). After a 30-min incubation, the reaction was stopped by the addition of 100 mM hydrochloric acid. Reaction products were solubilized by adjusting the solution to 50% methanol. An identical assay with no enzyme added was performed for every reaction. Protein was removed by filtering through an Amicon Ultracel 10K membrane filter (Millipore), and the flow-through was analyzed by UPLC. The solvents used in this method were: solvent A, 0.1% trifluoroacetic acid, and solvent B, 100% acetonitrile. Samples were analyzed using a method with an initial concentration of 10% B, followed by a 15-min linear gradient to 60% B, held for 1 min, then a 1-min linear gradient to 100% B, held for 1 min, and a 1-min linear gradient to the initial 10% B, held for 2 min, with a constant flow rate of 0.3 ml/min. Eluting compounds were detected at 280 nm and 340 nm. Enzyme activity was also determined for the reverse reaction, using authentic sinapyl *p*-coumarate **3Sa** or *p*-coumaryl *p*-coumarate **3Ha** and coenzyme A as substrates, with all other assay conditions as mentioned above. Standards for each of the substrates along with chemically synthesized standards of each monolignol conjugate **3** were used to determine retention times for each compound and identify HPLC chromatogram peaks. Crude reaction products isolated from the enzymatic reaction of sinapyl alcohol **1S** and *p*-coumaroyl-CoA **2a**, catalyzed by PMT, were identified by comparison with the synthetic standard peaks in proton NMR spectra and matching correlations in two-dimensional COSY NMR spectra.

One-dimensional Proton and Two-dimensional COSY NMR—NMR spectra of synthesized compounds and the crude reaction products from PMT reactions, dissolved in acetone-d$_6$, were acquired using standard pulse experiments and conditions on a Bruker Biospin (Billerica, MA) AVANCE 500 (500 MHz) spectrometer fitted with a cryogenically cooled 5-mm TCI gradient probe with inverse geometry (proton coils closest to the sample). Spectral processing used Bruker Topspin 3.1 (Mac) software. The central solvent peaks were used as internal reference (δ_H/δ_C 2.04/29.8). Standard Bruker implementations were used for one- and two-dimensional (gradient-selected multiple-quantum-filtered COSY, Bruker pulse program "cosygpmfqf" with gradients strengths (ratio 16:12:40) selected for a double quantum filter) spectra. HSQC and HMBC experiments were also used as usual for routine structural assignments of synthesized compounds. The COSY experiments shown in Fig. 5 used the following parameters: acquired from 10 to 0 ppm in both dimensions, in F2 (^{1}H) with 2000 data points (acquisition time 205 ms), and in F1 (^{1}H) with 256 increments (F1 acquisition time 25.6 ms) of 1 scan (for standards) or 4 scans for the crude PMT product, with a 1-s interscan delay. Processing used simple unshifted sine-bell apodization in both dimensions and benefited from one level of linear prediction (32 coefficients) in F1.

Kinetics—Kinetic analyses were performed using an assay modified from Santoro *et al.* (40). The standard 100-μl reaction mixture contained 50 mM sodium phosphate, pH 6, 2 mM DTNB, 0.01–1 mM CoA thioester substrate, 0.25–2.5 mM acetyl-CoA, and 0.005–1.0 mM monolignol alcohol substrate and initiated by adding 100 ng of purified OsPMT protein in 1$\times$ BSA (NEB). The CoA thioester substrates included *p*-coumaroyl-CoA **2a** and caffeoyl-CoA **2b**, and the monolignol substrates included sinapyl alcohol **1S** and *p*-coumaryl alcohol **1H**. Enzyme activity was measured as an increase in CoASH, detected with DTNB at A_{412}, which is released as a result of monolignol conjugate synthesis (40). The absorbance was measured every 3 min for 40 min on a Spectramax Plus microplate reader using Softmax Pro 5.3 (Molecular Devices). The reactions were stopped by adding hydrochloric acid to a concentration of 100 mM and then solubilized by adding methanol to 50%. Aliquots of 10 μl from each assay were analyzed via UPLC to verify product production. A standard curve was created for each CoA thioester from triplicate assays of five concentrations from 50 nM to 1 mM of coenzyme A. Each reaction contained the same buffer and DTNB concentrations as the kinetic assays, along with 0.5 mM CoA thioester (*p*-coumaroyl-CoA **2a**, caffeoyl-CoA **2b**, feruloyl-CoA **2c** or acetyl-CoA). The equation derived from fitting this standard curve was used to calculate the quantity (moles) of product synthesized in the assay. Kinetic parameters, V_{max} and K_m, were calculated using a nonlinear regression by entering the reaction rate and substrate concentration into the program R64, version 2.12.0 (41).

Phylogenetic Analysis—We obtained sequences closely related to OsPMT from plant species having sequenced genomes using the Phytozome 7 locus keyword search feature (42). We aligned these sequences using the program MUSCLE and generated a phylogenetic tree with the program TREE-PUZZLE (Fig. 3) (43, 44). TREE-PUZZLE uses quartet-puzzling to find a candidate maximum-likelihood tree. This involves calculating maximum-likelihood values for all groups of four sequences,
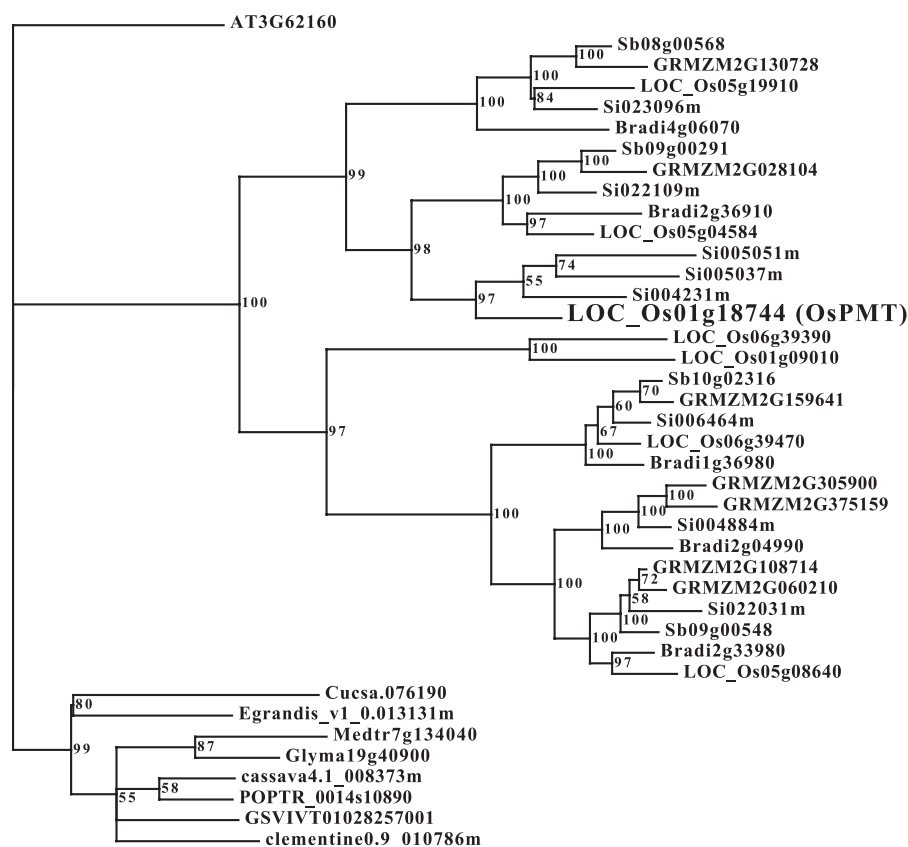
FIGURE 3. **Phylogentic tree of H***xxx***D acyltransferases related to OsPMT1.** Angiosperm sequences related to OsPMT (*bold*) were obtained using Phytozome 7 and aligned using the multiple sequence alignment program MUSCLE 3.8.31. The resulting alignment was input into the program TREE-PUZZLE 5.2 with default settings to produce a phylogenetic tree. A dendrogram was produced using the program Dendroscope (36, 42).

and these values are used to generate intermediate trees. It is not possible to generate all possible trees, and the generation of the intermediate trees is affected by the order of sequences in the alignment. TREE-PUZZLE was set to repeat the generation of intermediate trees 10,000 times with different ordering of sequences. The presented tree is the majority-rule consensus tree. Each branch is labeled with the percentage of intermediate trees supporting that branch. Trees were displayed using the program Dendroscope (45).

RESULTS

Identification of Candidate Gene—The most likely class of enzymes to catalyze such a reaction belong to the BAHD acyl-transferases, currently referred to as H*xxx*D acyltransferases, as they catalyze many similar reactions. Because *p*-coumaroyla-tion is a distinctive feature of grass lignins, we reasoned that a grass specific H*xxx*D acyltransferase that is co-expressed with genes involved in monolignol biosynthesis would be a good candidate for the enzyme responsible for acylation of monoli-gnols. We used the RiceXPro data base version 1.5 co-expres-sion tool at the National Institute of Agrobiological Sciences Genome Resource Center (Ibaraki, Japan) to identify H*xxx*D acyltransferases co-expressed with each of the three 4CL genes in rice (46, 47). The 4CL enzyme is required for the synthesis of lignin monomers, and the most highly correlated gene with 4CL (Os08g0245200) is LOC_Os01g18744, an H*xxx*D acyl-transferase hereafter referred to as OsPMT (or simply as PMT;
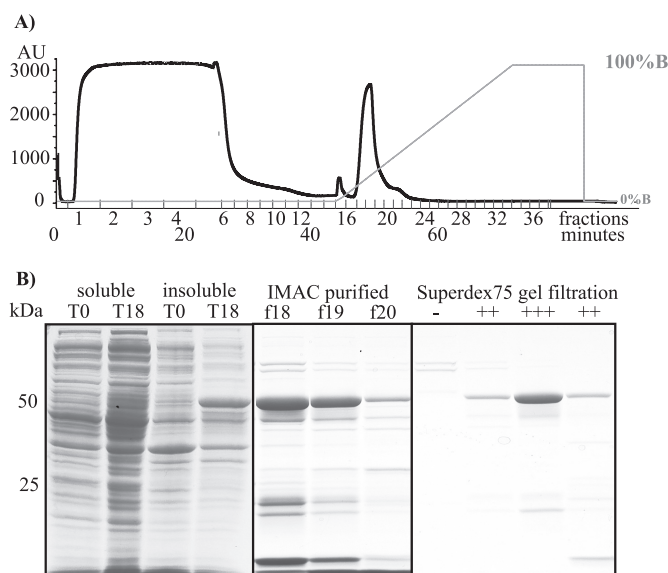
FIGURE 4. **Heterologous expression of PMT in *E. coli*.** *A*, FPLC chromato-gram showing IMAC purification of expressed soluble PMT from *E. coli* repre-sented in *black*, the buffer gradient represented in *gray*, and collected frac-tions below. *B*, SDS-PAGE showing soluble and insoluble protein fractions from *E. coli* at induction of PMT, T0, and after 18 h of induction, T18, IMAC fractions 18–20, and Superdex 75 gel filtration fractions assayed for PMT enzyme activity. Fractions labeled with + indicate fractions with PMT activity; − indicates no activity measured.

see supplemental Fig. 1). OsPMT is also correlated with several other biosynthetic enzymes from the lignin pathway including the rice caffeic acid 3-*O*-methyltransferase (COMT), caffeoyl-
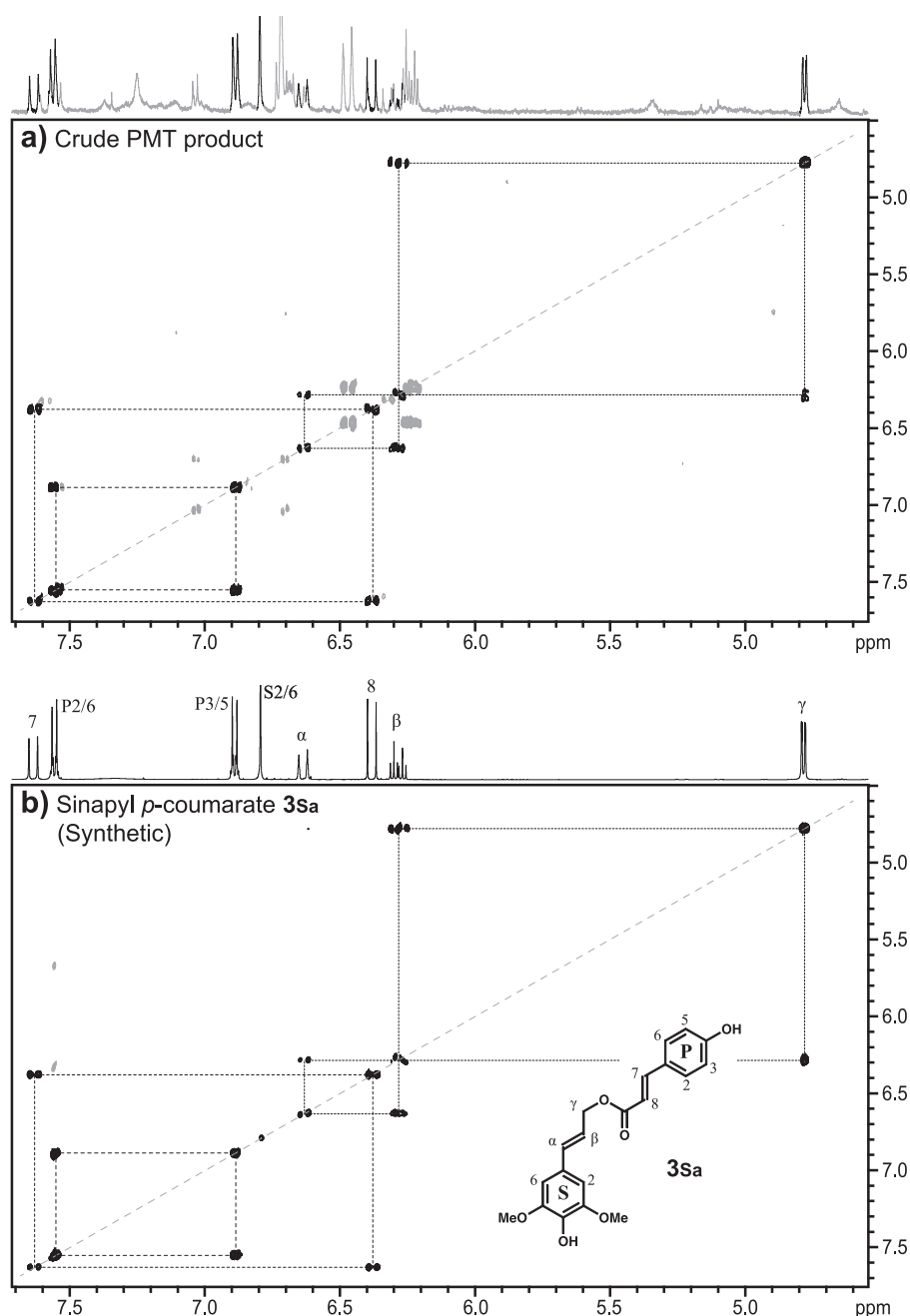
FIGURE 5. **PMT-catalyzed reaction between sinapyl alcohol 1S and *p*-coumaroyl-CoA 2a produced the sinapyl *p*-coumarate conjugate 3Sa as authenticated by one-dimensional proton (*horizontal* projection) and two-dimensional COSY NMR.** *a*, crude PMT product contains sinapyl *p*-coumarate **3Sa** as a major product, as determined by comparison of its proton and two-dimensional COSY NMR spectra (*solid black lines*) with *b*, authentic (synthetic) sinapyl *p*-coumarate **3Sa**.

CoA *O*-methyltransferase (CCoAOMT), and phenylalanine ammonia-lyase, along with several peroxidases, cell death-related and senescence-related genes (supplemental Table I). A phylogenetic analysis indicates that OsPMT is in a grass-specific group (36, 42– 45). Because OsPMT is a grass-specific H*xxx*D acyltransferase co-expressed with 4CL, we chose this gene for further study.

Expression of OsPMT in E. coli—A synthetic gene having the amino acid sequence for OsPMT, with an N-terminal His tag, was expressed in *E. coli* BL21 cells. This protein was purified using IMAC followed by size exclusion chromatography (Fig. 4*A*). OsPMT protein expression and purification were moni-

tored throughout this process by SDS-PAGE by following a protein near the expected molecular mass of 47 kDa (Fig. 4*B*). The identity of this protein was verified as OsPMT by LC-MS/MS on in-gel trypsin digested peptides. The additional bands present in the Superdex 75 fraction were identified as fragments of OsPMT by LC-MS/MS.

Determination of OsPMT Kinetic Parameters—Purified OsPMT produced a compound that eluted with authentic sinapyl *p*-coumarate **3Sa** when incubated with sinapyl alcohol **1S** and *p*-coumaroyl-CoA **2a**. This activity followed the OsPMT protein during gel permeation chromatography as shown in Fig. 4*A*. The identity of the product was shown to be sinapyl

TABLE 1

Kinetic data for OsPMT purified from *E. coli* extracts

K_m and V_{max} data were calculated from the mean of at least three replicates $\pm$ S.E. 1 pkat = 1 pmol of substrate s^{-1}. NA indicates parameters not calculated due to low activity.

| Varying substrate	Saturating substrate	$K_m \pm$ S.E.	$V_{max} \pm$ S.E.	K_{cat}	K_{cat}/K_m
		μM	$pkat\ mg^{-1}$	s^{-1}	$mM^{-1}\ s^{-1}$
Sinapyl alcohol **1S**	*p*-Coumaroyl-CoA **2a**	35 $\pm$ 5	10,800 $\pm$ 351	0.51	14.6
p-Coumaroyl-CoA **2a**	Sinapyl alcohol **1S**	105 $\pm$ 12	12,500 $\pm$ 417	0.60	5.7
p-Coumaryl alcohol **1H**	*p*-Coumaroyl-CoA **2a**	141 $\pm$ 14	54,200 $\pm$ 2,080	2.58	18.3
p-Coumaroyl-CoA **2a**	*p*-Coumaryl alcohol **1H**	281 $\pm$ 62	61,500 $\pm$ 5,300	2.93	10.4
p-Coumaroyl-CoA **2a**	Coniferyl alcohol **1G**	NA	<2,180	NA	NA
Sinapyl alcohol **1S**	Caffeoyl-CoA **2b**	15 $\pm$ 2	8,100 $\pm$ 244	0.39	25.7
Caffeoyl-CoA **2b**	Sinapyl alcohol **1S**	75 $\pm$ 5	7,500 $\pm$ 150	0.36	4.7
p-Coumaryl alcohol **1H**	Caffeoyl-CoA **2b**	27 $\pm$ 6	5910 $\pm$ 399	0.28	10.4
Caffeoyl-CoA **2b**	*p*-Coumaryl alcohol **1H**	92 $\pm$ 11	8,590 $\pm$ 309	0.41	4.4
Caffeoyl-CoA **2b**	Coniferyl alcohol **1G**	NA	<2,980	NA	NA
Feruloyl-CoA **2c**	Sinapyl alcohol **1S**	NA	<1,230	NA	NA
Feruloyl-CoA **2c**	*p*-Coumaryl alcohol **1H**	NA	NA	NA	NA
Feruloyl-CoA **2c**	Coniferyl alcohol **1G**	NA	NA	NA	NA
Acetyl-CoA	Sinapyl alcohol **1S**	489 $\pm$ 56	1,683 $\pm$ 64	0.08	0.2
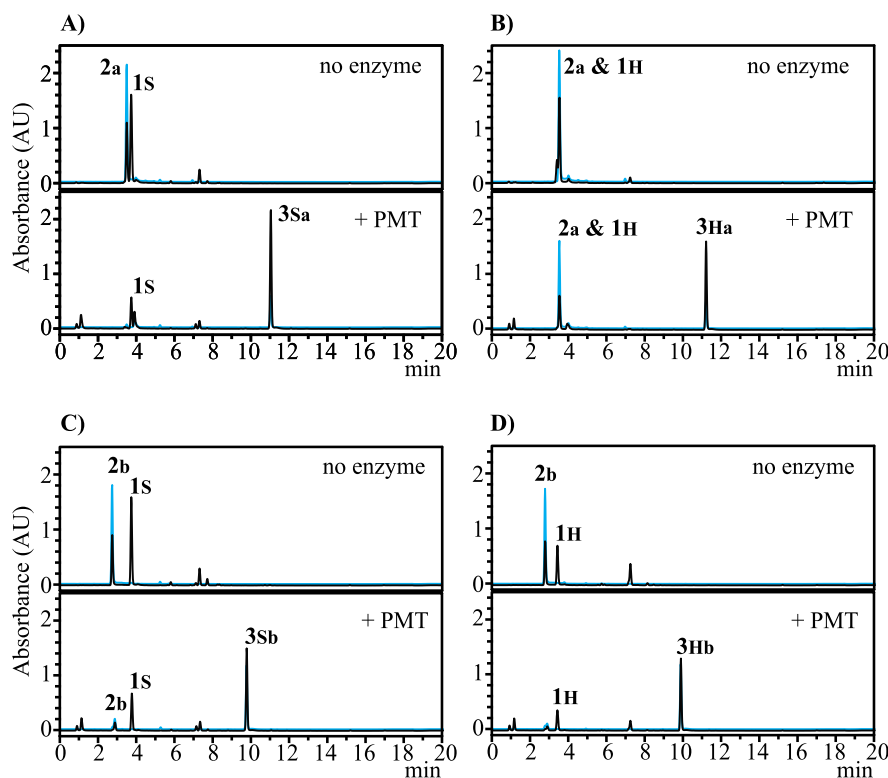
FIGURE 6. HPLC analysis of PMT enzyme assays. *A–D*, HPLC chromatograms of PMT enzyme assay with no enzyme and with purified OsPMT (+ PMT), UV absorbance monitored at 280 nM (*black*) and 340 (*blue*), of the following reactions: *p*-coumaroyl-CoA **2a** with sinapyl alcohol **1S** and making sinapyl *p*-coumarate **3Sa** (*A*); *p*-coumaroyl-CoA **2a** with *p*-coumaryl alcohol **1H** making *p*-coumaryl *p*-coumarate **3Ha** (*B*); caffeoyl-CoA **2b** with sinapyl alcohol **1S** making sinapyl caffeate **3Sb** (*C*); and caffeoyl-CoA **2b** with *p*-coumaryl alcohol **1H** making *p*-coumaryl caffeate **3Hb** (*D*).

p-coumarate **3Sa** by NMR (Fig. 5). Enzyme substrate specificity was examined for the acyl donors: *p*-coumaroyl-CoA **2a**, caffeoyl-CoA **2b**, feruloyl-CoA **2c**, and acetyl-CoA, and the acyl acceptors *p*-coumaryl alcohol **1H**, coniferyl alcohol **1G**, and sinapyl alcohol **1S**. Of the tested acyl donors *p*-coumaroyl-CoA **2a** and caffeoyl-CoA **2b** were good substrates whereas feruloyl-CoA **2c** and acetyl-CoA were poor substrates (Table 1). The enzyme had the highest affinity for sinapyl alcohol **1S**, but the synthetic rate was six times higher with *p*-coumaryl alcohol **1H**. We were not able to establish kinetic parameters for caffeyl alcohol **1C** due to its limited solubility; we consider this to be relatively unimportant because caffeyl alcohol has never been found incorporated into monocot or dicot lignins and has in

fact only recently been identified in a softwood down-regulated in CCoAOMT (48). Too little activity was observed with feruloyl-CoA **2c** or coniferyl alcohol **1G** as the acceptors to obtain the K_m for these compounds, but we did obtain an estimate of the maximum velocity. Both the relatively low maximum velocity and the high K_m measured for acetyl-CoA suggest that it is a poor substrate for this enzyme. The activity measured with *p*-coumaroyl-CoA **2a** or caffeoyl-CoA **2b** as the acyl donor and coniferyl alcohol **1G** as the receptor was also noticeably less than that of sinapyl alcohol **1S** and *p*-coumaryl alcohol **1H**. OsPMT was able to efficiently synthesize sinapyl *p*-coumarate **3Sa**, *p*-coumaryl *p*-coumarate **3Ha**, sinapyl caffeate **3Sb**, and *p*-coumaryl caffeate **3Hb** as measured by HPLC products from

enzyme assay reactions (Fig. 6). Complete kinetic properties were determined for these substrates using a method modified from Santoro *et al.* (40). Control reactions with no acyl donor substrate were run for each acyl acceptor and showed no OsPMT activity. These controls were repeated for each acyl donor substrate, containing no acyl acceptor, and also showed no activity. Reactions containing no enzyme produced no OsPMT activity (Fig. 6).

The kinetic properties indicate that OsPMT has similar affinity for sinapyl alcohol **1S** and *p*-coumaryl alcohol **1H**, shown by the very similar K_m values; however, the reaction rates vary with the acyl donor. Although the K_m values for *p*-coumaroyl-CoA **2a** and caffeoyl-CoA **2b** are similar, the maximum reaction rate for *p*-coumaroyl-CoA **2a** is at least 5-fold higher. The highest catalytic efficiencies were measured for sinapyl **1S** and *p*-coumaryl alcohols **1H** and for *p*-coumaroyl-CoA **2a**. OsPMT appears to synthesize primarily *p*-coumaryl *p*-coumarate **3Ha** and sinapyl *p*-coumarate **3Sa**. Based on the kinetic data, if *p*-coumaryl alcohol **1H** is the more abundant monolignol, *p*-coumaryl *p*-coumarate **3Ha** will be produced. If sinapyl alcohol **1S** concentrations are greater or similar, the enzyme will produce sinapyl *p*-coumarate **3Sa**.

DISCUSSION

Lignocellulosic biomass represents an abundant, inexpensive, and locally available feedstock for conversion to biofuels. However, the complex structure of lignin, including the ether and carbon-carbon bonds that bind together the various subunits of lignin, and its cross-linking with other plant cell wall polymers, make it the most important factor in the recalcitrance of plant cell walls against polysaccharide utilization. Gaining access to the carbohydrate polymers of plant cell walls for use as carbon and energy sources therefore requires significant energy inputs and harsh chemical treatments. Plants with altered lignin structures that could be more readily cleaved under milder conditions would reduce the costs of papermaking and make the production of biofuels more competitive with the currently existing procedures for producing oil and gas fuels. Currently, we do not understand the role of lignin acylation in wall recalcitrance. The identification of enzymes responsible for such acylation allows for the manipulation of acylation levels in plants to assess the importance of this modification. These genes may also enable the production of plants producing altered lignins and may assist our efforts to produce new conjugates (24).

The transferase enzyme OsPMT expressed in *E. coli* was shown to catalyze transesterification reactions between monolignols **1** and *p*-coumaroyl-CoA **2a** (Fig. 1), producing primarily monolignol *p*-coumarates **3Xa** (where X = **H**, **C**, **G**, or **S**, Fig. 1). Although activity is measured using caffeoyl-CoA **2b** as well, the catalytic efficiency indicates that this enzyme has a higher affinity for *p*-coumaroyl-CoA **2a**. Kinetic data also indicate that the affinity for sinapyl alcohol **1S** is high; however the reaction rate and catalytic efficiency for *p*-coumaryl alcohol **1H** with saturating *p*-coumaroyl-CoA **2a** suggest that OsPMT will produce more *p*-coumaryl *p*-coumarate **3Ha** if local concentrations of *p*-coumaryl alcohol are high enough. Thus, OsPMT could be the enzyme responsible for the *p*-coumaroylation seen

in grasses. Because of the high *p*-coumaroylation, seen primarily on syringyl lignin units **S** and the low concentrations of *p*-hydroxyphenyl **H** units in grass lignins, we expect the preferred substrates for the OsPMT reaction in the plant to be sinapyl alcohol **1S** and *p*-coumaroyl-CoA **2a**. The enzyme favors the synthesis of sinapyl *p*-coumarate **3Sa** over coniferyl *p*-coumarate **3Ga**, which is consistent with the ratio (~90:10) of these conjugates observed incorporated into grass cell walls. The facility of OsPMT to synthesize *p*-coumaryl *p*-coumarate **3Ha** raises the possibility that grasses may use this compound in the synthesis of monolignols. The well established pathway includes the transesterification of *p*-coumaroyl-CoA **2a** to a shikimic acid ester, which is the substrate for C3H (Fig. 2). Given the finding reported here it will be interesting to see whether *p*-coumaryl *p*-coumarate **3Ha** is a substrate for C3H as this could represent an alternative pathway for monolignol biosynthesis. The exact function of this enzyme awaits gene knockouts and overexpression in a grass species such as *O. sativa* or *Brachypodium distachyon*.

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